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(54) Title: METHOD OF SITE-SPECIFIC ALTERATION OF RNA AND PRODUCTION OF ENCODED POLYPEPTIDES (57) Abstract A method of site-directed alteration (removal or removal followed by replacement) of selected nucleotides in an RNA molecule, as well as to mixed phosphate backbone oligonucleotides useful in the method. It further relates to a method of producing polypeptides or proteins encoded by the RNA molecule altered by the present method. Through use of the present method, site-directed cleavage of an RNA molecule is effected, followed by excision of the selected or target segment of the RNA molecule.		

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METHOD OF SITE-SPECIFIC ALTERATION OF RNA AND
PRODUCTION OF ENCODED POLYPEPTIDES

Description

Background

05 Several aspects of contemporary molecular genetics
and biotechnology make it desirable to be able to produce
genetically-altered proteins. For example, mutated
protein domains are sometimes hyper-immunogenic,
facilitating the production of neutralizing antibody-
10 based vaccines. Moreover, site-directed mutations,
ideally one amino acid at a time, can be a powerful
approach to deciphering protein structure and/or enzyme-
substrate reaction mechanisms.

Typically, deletions or substitutions of amino acids
15 are made at the gene or DNA level, by recombinant DNA
techniques which rely on the use of restriction
endonucleases. However, restriction endonucleases
available have a limited array of target sites in DNA
(usually palindromic hexanucleotide or octanucleotide
20 sequences). Deletion of a particular in-frame trinucleo-
tide or trinucleotides may not be possible because there
may be no suitably located restriction sites. As a
result, presently-available methods of altering an amino

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acid sequence by altering the DNA sequence which encodes it, are limited in their applicability.

Summary of the Invention

05 The present invention relates to a method of site-directed alteration (removal or removal followed by replacement) of selected nucleotides in an RNA molecule, as well as to mixed phosphate backbone oligonucleotides useful in the method. It further relates to a method of producing polypeptides or proteins encoded by the RNA
10 molecule altered by the present method. Through use of the present method, site-directed cleavage of an RNA molecule is effected, followed by excision of the selected or target segment of the RNA molecule. Following cleavage and excision, in one embodiment, the
15 two segments of the resulting interrupted RNA molecule are joined, through the action of an appropriate ligase. This results in production of a continuous RNA molecule, referred to as an altered RNA molecule, which is the same as the original RNA molecule except that it lacks the
20 nucleotides originally present in the target segment of the RNA molecule. In a second embodiment, selected nucleotides can be introduced into the space or gap created by removal of the target RNA segment; a continuous RNA molecule is created by ligating the
25 selected nucleotides introduced in this manner to the nucleotide on each side of the gap.

In the present method, an RNA molecule whose nucleotide sequence is to be altered in a site-directed manner

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is brought into contact with an oligonucleotide, referred to as a mixed phosphate backbone oligonucleotide, in the presence of RNase H. The mixed phosphate backbone oligonucleotide is complementary to all or a portion of an RNA molecule which includes a target segment to be altered. In addition, the mixed phosphate backbone oligonucleotide includes an internal portion or segment of deoxynucleotides which is capable of activating RNase H and is flanked on each side by a sequence of nucleotides which is unable to activate RNase H. The internal sequence includes two or more consecutive phosphodiester linkages, which may be unmodified or modified. The flanking sequences are modified deoxyribonucleotide or ribonucleotide sequences. It has been shown that when such a mixed phosphate backbone oligonucleotide is contacted with a target segment of an RNA molecule, according to the method of the present invention, the result is RNase H mediated excision of RNA target nucleotides complementary only to the internal sequence of oligonucleotides. This makes it possible to excise precisely any desired nucleotide or oligonucleotide from an RNA molecule. Followed by RNA ligation, this results in a desired altered messenger RNA or other type of RNA. Thus, for the first time, it is possible to carry out precise excision of a selected segment of an RNA molecule.

As a result, it is possible to selectively delete any desired number of nucleotides and, if desired, to introduce replacement nucleotides. The encoded amino acid sequence or polypeptide can be produced by expressing the altered RNA in vitro or in vivo. As a result, a

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selected amino acid sequence or selected polypeptide can be produced by the present method by: 1) producing an altered RNA molecule encoding a selected amino acid sequence or selected polypeptide and 2) expressing the altered RNA molecule under appropriate conditions. Cell-free translation of the altered RNA molecule can be carried out to produce desired mutant proteins useful, for example, for studies of protein structure or function. Alternatively, an appropriate mixed phosphate backbone oligonucleotide can be taken up by or introduced into cultured cells or into cells of an animal or a plant, in which endogenous RNase H and RNA ligase activities can produce altered RNAs; upon translation, corresponding genetically altered proteins are produced. This is useful, for example, as a means of producing defective viral or infectious/pathogenic agent replication or gene expression, which can be useful therapeutically or prophylactically.

Brief Description of the Drawings

Figure 1 is a schematic representation of the present method for excision of a targeted segment of an RNA molecule in which sequence I is RNA from which a targeted segment is to be excised and sequence II is a mixed phosphate backbone oligonucleotide. Subscript "m" designates an RNase H-resistant internucleoside phosphate; subscript "s" indicates an RNase H-susceptible internucleoside phosphate; "X" designates any of the four ribonucleotides A, C, G or U; and "Y" designates a deoxyribonucleotide complementary to the ribonucleotide directly above it.

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Figure 2 is a schematic representation of repair of a genetic defect by the method of the present invention, in which IVa and IVb represent a double-stranded hybrid consisting of a smaller unmodified ribonucleotide (IVa) hybridized to a larger modified, RNase H-resistant deoxyribonucleotide (IVb) and V is the repaired RNA hybridized to the RNase H-resistant deoxyribonucleotide.

Detailed Description of the Invention

The present invention relates to a method of site-specific or site-directed alteration (removal or removal followed by replacement) of selected nucleotides (i.e., a target segment) in an RNA molecule, to produce an altered RNA sequence, as well as to mixed phosphate backbone oligonucleotides useful in the method. It further relates to a method of producing altered amino acid sequences or polypeptides by translating the altered RNA sequence, which results in production of the encoded molecule.

In the present method, a selected or target segment of an RNA molecule, such as pre-mRNA, mRNA or viral RNA, is altered as follows: an RNA molecule which includes the target segment (i.e., a nucleotide or a nucleotide sequence to be altered) is combined with an appropriately-selected mixed phosphate backbone oligonucleotide in the presence of RNase H. The mixed phosphate backbone oligonucleotide is complementary to all or a portion of the RNA molecule which includes the target RNA segment; it is of sufficient length to hybridize to the target RNA segment and sequences on either side and remain

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hybridized under the conditions used. The mixed phosphate backbone oligonucleotide has two key components: an internal portion or segment of deoxynucleotides which is capable of activating RNase H and two nucleotide sequences, which flank the internal segment, which are unable to activate RNase H. The internal deoxynucleotide segment includes two or more phosphodiester linkages, which may be unmodified or modified. The flanking nucleotide sequences may be deoxyribonucleotide or ribonucleotide sequences and is modified. That is, some or all of the internucleoside bridging phosphate residues are modified phosphates, such as methyl phosphonates, phosphoromorpholidates, phosphoropiperzaidates and phosphoramidates. An essential feature of the mixed phosphate backbone oligonucleotide is that the internal complementary segment is RNase H activating and the flanking complementary sequences are unable to activate RNase H.

As demonstrated herein, site-directed alteration occurs when the RNA molecule which includes the target segment and an appropriately selected mixed phosphate backbone oligonucleotide are combined in the presence of RNase H and maintained under appropriate conditions (e.g., temperature, time, salt concentration) for complementary nucleotide sequences to hybridize and RNase H to be activated (i.e., to be able to cleave and excise). That is, as a result, the nucleotides in the RNA molecule to which the internal segment of the mixed phosphate backbone oligonucleotide is complementary are excised precisely from the RNA molecule.

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In one embodiment of the present method, the gap resulting from excision of the target RNA segment can be closed by the activity of an appropriate ligase(s), resulting in a continuous RNA molecule referred to as an altered RNA molecule. The resulting altered RNA molecule differs from the RNA molecule only as to the target segment, which is not present in the altered RNA molecule.

In a second embodiment the gap created by the action of RNase H as described above can be filled in by introducing a segment of replacement nucleotides, which can be of any length appropriate to fit into the gap created in the RNA molecule. The segment of replacement nucleotides is subsequently linked to the adjacent nucleotides of the RNA molecule by an appropriately selected ligase(s). The nucleotide present at each end of the segment of replacement nucleotides is ligated to the nucleotide present on the respective "end" of the gap created in the RNA molecule (underlined in Figure 2(IIIa)). The resulting altered RNA molecule differs from the RNA molecule in that the target segment (present in the RNA molecule) has been removed and a replacement nucleotide sequence introduced in its place.

Altered RNA produced as described herein can be expressed, either in vitro or in vivo, to produce the encoded polypeptide or protein; as used herein, the term polypeptide includes proteins. For example, the altered RNA can be introduced into an appropriate vector, which is in turn introduced into a host cell capable of translating the altered RNA molecule and producing the encoded polypeptide. Polypeptides produced in this manner can be

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used for assessment of their structural/functional characteristics and a comparative assessment of polypeptides which differ in a defined manner (e.g., by selected amino acids). Such polypeptides can also be used
05 therapeutically or prophylactically.

The following is a description, with reference to the figures, of two embodiments of the present method of site-directed alteration of an RNA molecule: a first
10 embodiment in which a selected nucleotide sequence (target segment) is removed and the resulting fragments ligated to produce an altered RNA molecule lacking the target segment and a second embodiment in which a
15 selected nucleotide sequence is removed and replaced by a selected nucleotide or nucleotides, which are ligated to the nucleotide at each side of the gap created by removal of the target RNA segment.

Method for Excising a Specific Small Segment of Ribonucleotides from an RNA Molecule

Figure 1 is a schematic representation of an embodiment of the present method. The sequence designated I is
20 a segment of an RNA molecule, which includes a target segment (UGACGUCA) to be altered. The RNA may be a pre-RNA, a viral RNA, or one of a variety of RNAs present in animal, plant or bacterial cells. The sequence
25 designated II is a segment of DNA which is a mixed phosphate backbone oligodeoxynucleotide complementary to the RNA molecule I. It can be synthesized using known techniques, such as chemical or enzymatic methods. The mixed phosphate backbone oligodeoxynucleotide includes an
30 internal sequence which is capable of activating RNase H

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and is flanked on each side by a DNA segment which is incapable of activating RNase H. The total length of the mixed phosphate backbone oligodeoxynucleotides varies, depending on the length of the target RNA segment to be altered, but must be sufficient to hybridize to the RNA molecule containing the target RNA segment and remain hybridized under the conditions used. The internal segment must be of sufficient length--at least two nucleotides--to be capable of activating RNase H, as demonstrated herein and by others (Walder, R.Y. and J.A. Walder, Proc. Natl. Acad. Sci. USA, 85:5011-5015 (1988); Furdon, P.J. et al., Nucleic Acids Res., 17:9193-9204 (1989)).

In the internal segment, the internucleoside bridging phosphate residues may be unmodified phosphates or any phosphate modification capable of activating RNase H, such as phosphorothioates. The flanking nucleotide sequences can be deoxyribonucleotides, as described in this embodiment or can be ribonucleotides and their modifications. The flanking sequences are connected by methyl phosphonates (PC), phosphoromorpholidates (PM), phosphoropiperazidates, phosphoramidates, or other modifications of internucleoside phosphates which are not able to activate RNase H. The RNA molecule I and the modified backbone oligodeoxynucleotide II are combined in the presence of RNase H, such as endogenous RNase H in a cell, and excision of the target segment occurs. A hairpin loop may form by pairing of complementary nucleotides, with the result that the two newly-formed segments are brought into proximity to each other. It may not be necessary that a hairpin loop form, however, in order for

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the segments produced by the activity of RNase H to be brought together because the two RNA segments are tethered by their base-pairing with the mixed phosphate backbone oligodeoxynucleotide. In the presence of an appropriate ligase(s), such as an endogenous ligase(s), the two newly-created segments are joined, by ligation of the nucleotide (underlined in Figure 1) on each side of the gap.

The definition of "activating RNase H" is based on the induced-fit theory of Koshland, in which "enzyme sites were envisaged as somewhat flexible and undefined before binding occurred, locking active site residues into defined positions around the substrate" (Koshland, D.E., Jr., Proc. Natl. Acad. Sci. USA, 44:98 (1958); Zeffren, E. and P.L. Hall, The Study of Enzyme Mechanisms, p. 201, John Wiley and Sons, New York, (1973)).

Method for excising and replacing a specific small segment of ribonucleotides from an RNA molecule

A second embodiment of the present invention is represented schematically in Figure 2. This embodiment is useful, as described below, in repairing a genetic defect by removing the defective nucleotides and replacing them with others, such as those present in the normal RNA or those which result in RNA encoding a desired polypeptide. As shown, an RNA molecule to be altered, such as pre-mRNA or mRNA in which a defect is present, is combined with an appropriate mixed phosphate backbone oligodeoxyribonucleotide in which the internal segment is capable of activating RNase H and the flanking nucleotide sequences are unable to activate RNase H, in the presence

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of RNase H. As described above, the target segment is excised in a site-directed manner, producing a split RNA molecule, as represented in Figure 2 as sequence IIIa. In this embodiment, a replacement oligomer, which is the series of nucleotides to be inserted into the gap created by excision of the target segment, is introduced into cells. Through hybridization exchange (as described in detail below), the replacement oligomer is introduced into the gap created as a result of the RNase H activity. Expression of the resulting altered RNA (which includes the desired/nondefective sequence in place of the defective sequence) results in production of the desired/nondefective polypeptide.

Certain genetic disorders (inborn errors of metabolism) can be corrected by the method of the present invention. For example, cystic fibrosis is usually due to a gene mutation in which a specific phenylalanine codon is deleted (Riordon, J.R. et al., Science, 245: 1066-1073 (1989)). Through use of the present method, the mutant cystic fibrosis mRNA present in an individual's cells can be cleaved at the missing codon by site-directed RNase H alteration, as described below. Introduction into the individual's cells of the appropriate oligoribonucleotide (i.e., one encoding a phenylalanine), followed by endogenous RNA ligase activity results in ligation and, thus, production of a wild-type mRNA encoding a normal protein product.

Other genetic defects can be remedied in a similar manner. Examples of genetic defects for which this would be appropriate are: the substitution of serine for glycine 844 in a severe variant of osteogenesis

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imperfecta (Pack, M. et al., J. Biol. Chem., 264:
19694-19699 (1989)); a glycine to serine substitution in
pro α (II) collagen in a form of dwarfism (Vissing, H. et
al., J. Biol. Chem., 264:18265-18267 (1989)); a glycine
05 833 conversion to aspartate in a mild variant of
Ehlers-Danlos syndrome IV (Tromp, G. et al., J. Biol.
Chem., 264:19313-19317 (1989)); an A to G transition in
an initiation codon mutation in the Apo C-II gene of a
patient with a deficiency of apolipoprotein C-II (Fojo.
10 S.S. et al., J. Biol. Chem., 264:10839-10842 (1989)); and
an aberrant GT splice-donor signal flanking exon 19 in
retinoblastoma RB-88 (Yandell, D.W. et al., N.E. J. Med.,
321:1689-1695 (1989)). Such precise genetic defects can
be repaired at the RNA level by the method described.

15 Correction of the specific defect associated with cystic
fibrosis

Riordan et al. have found that in approximately 70%
of the cases of cystic fibrosis, a trinucleotide (TTT) is
missing from a segment of the gene of chromosome 7, as
20 compared with its normal, wild-type counterpart. Thus,
the RNA transcription product of TTT, namely UUU, is also
missing.

An approach to correcting the defect associated with
cystic fibrosis is as follows: a mixed phosphate
25 backbone oligodeoxyribonucleotide is synthesized, which
consists of an internal segment composed of either
unmodified bridging phosphodiester bonds, or of modified
ones, such as phosphorothioates, (or certain other
modifications) which are capable of activating RNase H.

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As an example, Figure 2(I) illustrates a section of pre-mRNA from cystic fibrosis in which the UUU trinucleotide is missing. The mixed phosphate backbone oligomer A-TAG-TAA-CCA-CAA-A is synthesized. It includes an internal segment, bearing the subscript "s", which includes the internucleotide phosphates (unmodified, phosphorothioate or other) which are capable of activating RNase H. The flanking segments have the subscript designation "m", which signifies that the internucleoside bridging phosphate modifications render this segment of DNA incapable of activating RNase H. These "m" modifications may be methyl phosphonates, phosphoromorpholidates, phosphoropiperazidates, phosphoro-n-butylamidates, or other modifications of the internucleoside phosphate which result in inability to activate RNase H. In Figure 2(II) is shown a synthetic oligodeoxynucleotide consisting of six internal deoxynucleotides (subscript s) capable of activating RNase H in the portion of RNA to which they are hybridized. These residues are flanked on both sides by oligodeoxynucleotides whose internucleoside bridging phosphate modifications are designated by subscript "m", to indicate that these segments of the synthetic oligomer are incapable of activating RNase H. RNase H activation requires a DNA-RNA hybrid in which at least 2 consecutive nucleotides of the DNA strand have RNase H sensitive internucleoside linkages, as demonstrated herein (See Exemplification) and by others. Walder R.Y. and J.A. Walder, Proc. Natl. Acad. Sci. USA, 85:5011-5015 (1988); Furdon, P.J. et al., Nucleic Acids Res., 17:9193-9204 (1989)). Alternatively the sequence designated II can be a central RNase H sensitive section,

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as described above, flanked by two segments of ribonucleotides (subscript r), one on either side of the internal segment. In this case, the oligomer of Figure 2(II) would be represented as follows:

- 05 A-UAG-TAA-CCA-CCA-A. Hybridized ribonucleotide oligomers do not activate RNase H; therefore, the above oligomer would also consist of RNase H sensitive and resistant segments.

- As is described above with reference to Figure 1,
10 the target segment is excised in a site-specific manner, resulting in an interrupted RNA molecule; in this case, AUC-GGU have been removed, producing a 6-nucleotide gap. For example, in an individual, the synthetic oligomer (II), dissolved in physiological saline solution (e.g.,
15 at a concentration of 1×10^{-5} - 1×10^{-8} M) is injected (e.g., intravenously) into an individual with cystic fibrosis. The oligomer would become distributed into various organs and tissues of the individual. Hybridization of the synthetic oligomer with pre-mRNA or mRNA at
20 the targeted site would occur, activation of RNase H would follow, and cleavage and excision of the six nucleotide segment of RNA would result.

- Subsequently, another construct, such as that designated IVa in Figure 2, is administered to the
25 individual in such a manner that it enters cells (e.g., by intravenous injection). This construct is a hybrid, partially double-stranded synthetic oligomer which consists of a ribonucleotide unmodified nonamer with a 5'-phosphate, hybridized to a 27-mer deoxyribonucleotide,
30 in which all of the residues are modified so as to be nuclease resistant.

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As diagrammed in Figure 2, a hybridization exchange occurs, in which the longer, double-stranded oligomer construction IV would replace the shorter oligomer IIIb (A-TAG-TAA-CCA-CAA-A) in hybridizing with the natural RNA (IIIa) at the critical, excised defective site shown in Figure 2(IIIa). The latter bears a six nucleotide gap; the two segments are held in place by the oligomer A-TAG-TAA-CCA-CAA-A. Due to the greater hybridization length of the hybrid shown in Figure 2(IVb), a hybridization exchange occurs and the latter oligomer construct replaces the A-TAG-TAA-CCA-CAA-A. The nonamer (IVa) fills in the gap left by the nucleotide excision. The complementary pattern of the oligomer construct with the cystic fibrosis RNA will separate its two segments sufficiently to permit a nine nucleotide segment to be inserted where a six segment nucleotide was removed. The 5' phosphate on this unmodified RNA nonamer will permit ATP and RNA ligase, both present endogenously, perhaps assisted by other intracellular factors, to make the two covalent phosphodiester bonds necessary to repair the excision defect. The final result is to insert a trinucleotide UUU, at the desired location into the RNA transcript of the cystic fibrosis patient's genome. Hybridization exchange occurs because of the greater hybridization association constant and higher T_m of the 18 complementary nucleotides in the oligomer construct as compared with that of the eight nucleotides in the A-TAG-TAA-CCA-CAA-A. It is also possible that a natural RNA repair process may fill in the gap, if the unmodified rebonucleotide nonamer is omitted, and just the oligomer

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TTA-TAG-TAG-AAA-CCA-CAA-AGG-AUA is used for the transhybridization and repair steps.

Mode of administration of modified backbone oligonucleotide

05 The manner in which modified backbone oligonucleotides are provided will depend on the context in which they are used (i.e., in vitro, in vivo).

10 The modified backbone oligonucleotide is generally dissolved in water or a suitable buffered medium, such as Dulbecco's medium, Eagle's medium, or a similar physiological saline medium, typically at a concentration of 10^{-5} to 10^{-8} molar. In the case of a tissue culture system, the dissolved oligomer is sterilized by filtration through a bacterial filter, and is added to the
15 other components of the tissue culture incubation medium. In the case of a seed, the oligomer is dissolved in water and added to the seeds spread on sterile filter paper inside a sterile covered glass or plastic dish. In the case of a plant, dissolved oligomer, in aqueous media, is
20 added to the soil or other nutrient material in which the plant is growing. In the case of an animal or man, the oligomer, dissolved in physiological saline, may be injected subcutaneously, intraperitoneally, intramuscularly, intravenously, or possible by capsule orally.

25 It has been shown that oligomers such as the above-described enter living cells, and are found in significant concentrations both in the cytoplasm and in the nucleus within minutes after administration (Zamecnik, P.C. et al., Proc. Natl. Acad. Sci. USA, 83:4143-4146
30 (1986); Goodchild, J. et al., Current Communications in

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Molecular Biology-Antisense RNA and DNA, Cold Spring Harbor, pp. 135-139 (1988); Wickstrom, E.L. et al., Proc. Natl. Acad. Sci. USA, 85:1028-1032 (1988)).

05 The following exemplification demonstrates that site-specific excision of nucleotides at the RNA level have been carried out, using the present method and modified backbone oligonucleotides as described herein.

EXEMPLIFICATION

Materials and Methods

10 Oligodeoxynucleotide Synthesis

Oligodeoxynucleotides were synthesized on an automated instrument (model 8700, Milligen, MA). Normal phosphodiester (PO) oligodeoxynucleotides and the analogous phosphorothioate (PS) or phosphoramidate
15 oligodeoxynucleotides were synthesized using H-phosphonate chemistry (Agrawal, S. et al., Proc. Natl. Acad. Sci. USA, 86:7790-7794 (1989); and Agrawal, S. et al., Proc. Natl. Acad. Sci. USA, 85:7079-7083 (1988)).

Oligodeoxynucleoside methylphosphonate (PC) analogues
20 were assembled by using nucleoside methylphosphonamidites (Agrawal, S. and J. Goodchild, Tetrahedron Lett., 28:3539-3542 (1987)). Oligodeoxynucleotides containing both PO and PC internucleoside linkages were assembled by using nucleoside- β -cyanoethylphosphoramidites and nucleoside
25 side methylphosphonamidites, and oligodeoxynucleotides containing both PC and PS linkages were synthesized from nucleoside methylphosphonamidites (Agrawal, S. and J. Goodchild, Tetrahedron Lett., 28:3539-3542 (1987)) and

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nucleoside H-phosphonates. Oligodeoxynucleotides containing both PO and phosphoramidate linkages were synthesized by using nucleoside- β -cyanoethylphosphoramidites and nucleoside H-phosphonates.

05 RNase H Assays

The 13-mer oligodeoxynucleotides complementary to nucleotides 2-14 of human U1 small nuclear RNA (counting the G cap as nucleotide (0)) were added at 100 μ g/ml to a HeLa cell nuclear extract (Dignam, J.D. *et al.*, Nucleic
10 Acids Res., 11:1475-1479 (1983)) containing 0.5 mM ATP, 20 mM creatine phosphate, 3.2 mM $MgCl_2$, and 1000 units of RNase per ml and were incubated under the conditions specified. RNA was isolated from the nuclear extract by phenol/chloroform extraction and ethanol precipitation,
15 followed by electrophoresis in a 10% polyacrylamide gel containing 8.3 M urea. The RNAs were visualized by ethidium bromide staining.

A second RNase H assay was also carried out in nuclear extracts by using an exogenous ^{32}P -labeled RNA.
20 A 514-nucleotide test RNA (hereafter termed "514 RNA" for convenience) was generated by SP6 RNA polymerase transcription of a HindIII-linearized pGEM-2 clone, pT7H β Δ 6. 514 RNA is antisense to the first two exons and intron of human β -globin pre-mRNA and was chosen for the reasons
25 described below. 514 RNA labeled with a [α - ^{32}P]GTP, [α - ^{32}P]CTP, and [α - ^{32}P]UTP was added to the nuclear extract containing the specified oligodeoxynucleotide at an oligomer-to-514 RNA molar ratio of 3000:1, unless
otherwise noted. After incubation as specified, RNA was
30 extracted and the ^{32}P -labeled 514 RNA or its cleavage

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products were visualized by electrophoresis and autoradiography.

Results

Action of RNase H on Oligodeoxynucleotide-U1 Small

05 Nuclear RNA Hybrids

HeLa cell nuclear extracts contain RNase H activity that can act on DNA-RNA hybrids that form after addition of oligodeoxynucleotides complementary to certain endogenous nuclear RNAs (Kraimer, A.R. and T. Maniatis, Cell, 10 42:725-736 (1985)). The ability of this RNase H activity to cleave the 5'-terminal nucleotides of endogenous U1 small nuclear RNA, after incubation of the various modified oligomers in the nuclear extract, was investigated. Incubation of the PO oligomer in the nuclear extract led 15 to cleavage of a large proportion of the U1 RNA to a product (U1*) having the mobility expected for removal of the first 15 nucleotides (cap, nucleotide 1, and nucleotides 2-14). There was a lack of effect on the mobility of any other RNAs present, demonstrating the 20 high sequence specificity of the U1 oligomer-directed RNase H cleavage. U1 cleavage was also observed with the PS oligomer, although to a consistently lesser extent than with the PO oligomer. In contrast, no cleavage was observed with the PC, phosphoroN-morpholidate (PM), or 25 phosphoro-N-butylamidate (PB) oligomers. Reducing the temperature of incubation to 20°C and extending the time to 60 minutes did not increase the extent of cleavage observed with the PS oligomer, nor did it reveal cleavages with the PC, PM or PB oligomers. The pattern

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of U1 cleavages seen with the various oligomers was also not altered by adding E. coli RNase H (Pharmacia, final concentration 8 units/ml) to the nuclear extract at the outset of incubation..

05 RNase H Action at an Internal RNA Site Hybridized with
Normal and Modified Oligodeoxynucleotides

The foregoing U1 RNA cleavages involve the 5' extremity of a small RNA. Indeed, the 5' end of U1 RNA is an exceptionally favored target for oligodeoxy-
10 nucleotide-directed RNase H cleavage since almost all the other regions of this RNA molecule are tightly complexed with proteins in the U1 small nuclear ribonucleoprotein particle (Patton, J.R. et al., Mol. Cell. Biol., 7: 4030-4037 (1987); Patton, J.R. et al., Proc. Natl. Acad.
15 Sci. USA, 85:747-751 (1988); Patton, J.R. et al., Mol. Cell. Biol., 9:3360-3368 (1989)). Therefore, the action of RNase H on hybrids formed by normal or modified oligomers at internal sites in a longer RNA was also investigated.

20 For this purpose a test RNA which is 514 nucleotides long (termed 514 RNA) was used. 514 RNA is antisense to the first two exons and intron of human β -globin pre-mRNA. The underlying reasoning was that, because it is antisense to a pre-mRNA, 514 RNA would not undergo
25 splicing when added to nuclear extract (the cleavage-ligation steps of which would otherwise complicate analysis of oligodeoxynucleotide-directed RNase H cleavages).

A series of normal and modified 15-mer oligodeoxy-
30 nucleotides complementary to nucleotides 349-363 of 514

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RNA was synthesized chemically (Table 1, oligomers A-E). This particular 514 RNA site was selected because oligomer-directed RNase H cleavage would generate two fragments of readily distinguishable lengths (150 and 348 nucleotides) and also because this 15-nucleotide sequence does not occur elsewhere in 514 RNA (Lawn, R.M. et al., Cell, 21:647-651 (1980)). Melting curves in 0.16 M Na⁺ for four of these oligodeoxynucleotides after duplex formation with the complementary (PO) oligomer revealed that the PO, PC, PS and PM oligomer-containing duplexes had t_m s of 53°C, 46°C, 43°C and 38°C, respectively, indicating a lower duplex stability for the modified oligomers, in part confirming previous reports (Stein, C.A. et al., Nucleic Acids Res., 16:3209-3221 (1988); Froehler, B. et al., Nucleic Acids Res., 16:4831-4839 (1988); Quartin, R.S. and J.G. Wetmur, Biochemistry, 28:1040-1047 (1989); Agrawal, S. et al., Nucleosides Nucleotides, 8:819-823 (1989)).

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TABLE 1

	<u>Oligomer</u>	<u>Sequence</u>	<u>Internucleoside</u>
			<u>Linkage</u>
05	A	GTA TCA AGG TTA CAA	PO
	B	GTA TCA AGG TTA CAA	PS
	C	GTA TCA AGG TTA CAA	PM
	D	GTA TCA AGG TTA CAA	PB
	E	GTA TCA AGG TTA CAA	PC
	F*	GTA TCA <u>TAT GAG ACA</u>	PO
10	G*	GTA <u>GCA</u> AGG <u>CTA</u> CAA	PO
	H*	GTA TGA <u>GAC ATA TAC</u>	PO

Underlined nucleotides indicate base pairing mismatches; PB is phospho-N-butylamidate.

The series of normal and modified oligodeoxy-nucleotides shown in Table 1 were incubated with ³²P-labeled 514 RNA in nuclear extracts, under the conditions specified in the Materials and Methods section, for either 30 minutes or 3.5 hours.

The results of incubating these oligomers with 514 RNA in HeLa nuclear extract are as follows: After 30 minutes of incubation without any oligomer, intact input 514 RNA was the only labeled species visualized. After 3.5 hours of incubation without any oligomer, the 514 RNA was completely degraded by the action of ribonuclease known to be present in the extract. Incubation of 514 RNA in the extract for 30 minutes together with either the PO or PS oligomers resulted in precise cleavage of the substrate RNA into two fragments of the sizes

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expected from the location of the oligomer-complementary sequence. Surprisingly, in the case of the PS oligomer, these two fragments were still present, albeit in slightly degraded form, after 3.5 hours of incubation.

05 Incubation of 514 RNA with PM oligomer for 30 minutes resulted in partial cleavage (see the figure legend for additional details). No cleavage was observed with the PB or PC oligomers under these conditions.

10 PO oligomers that were only partially complementary to 514 RNA (oligomers F, G and H in Table 1) were also tested. None of these oligomers, containing 4, 5 or 6 uninterrupted complementary nucleotides out of the 15 (Table 1), elicited RNase H cleavage of 514 RNA.

The finding that the PS oligomer was less effective
15 than the PO oligomer in eliciting RNase H cleavage in the U1 RNA assay raised the possibility that the more complete RNase H cleavages observed with both PO and PS oligomers in the 514 RNA assay might reflect the particular reaction conditions employed. Therefore, a
20 range of oligomer-to-514 RNA molar ratios tests (0.1:1-1000:1), all below that used in the above-described (3000:1), was investigated. Results showed that virtually complete 514 RNA cleavage occurred with the PO oligomer at an oligomer-to-RNA ratio of
25 100:1, whereas a comparable extent of 514 cleavage with the PS oligomer occurred at a oligomer-to-RNA ratio of 1000:1. A very similar, incomplete extent of cleavage was observed with the PO and PS oligomers at ratios of 10:1 and 100:1, respectively. The possibility that these
30 results might reflect a preferential instability of the PS oligomer during incubation in the nuclear extracts was

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examined by experiments in which either the PO or the PS oligomer was preincubated in nuclear extract for 30 minutes, followed by addition of ^{32}P -labeled 514 RNA and incubation for an additional 30 min. This revealed the same extent of difference in RNase H cleavage as described above.

"Restriction Endonuclease-Like" Cleavage with Oligomers Containing RNase H-Sensitive and -Resistant Internucleoside Linkages

10 The extreme differences between the RNase H sensitivity of DNA-RNA hybrids containing PO or PS oligodeoxynucleotides, contrasted with ones with PC, PM or PB oligomers, led to an investigation of how RNase H acts on a DNA-RNA hybrid in which only a small proportion of
15 internucleoside linkages in the DNA strand were RNase H-sensitive. Table 2 shows the series of oligomers that were synthesized to address this issue.

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TABLE 2

Oligomer	Sequence
I	AGG T
J	A AGG TT
05 K	<u>GTA TCA AGG TTA CAA</u>
L	<u>GTA TCA AGG TTA CAA</u>
M	<u>GTA TCA AGG TTA CAA</u>
N	<u>GTA TCA AGG TTA CAA</u>
O	<u>GTA TCA AGG TTA CAA</u>
10 P	<u>GTA TCA AGG TTA CAA</u>
Q	<u>GTA TCA AGG TTA CAA</u>
R	<u>GTA TCA AGG TTA CAA</u>

Underlined nucleotides are PC; dashed nucleotides are PS; Boxed nucleotides are phosphoromorpholidates; Double-underlined nucleotides are PM (oligomer Q) or PB; the remaining nucleotides are PO.

The oligomers listed were tested for their capacity to elicit RNase H action after hybridization to 514 RNA, as in the preceding experiments.

Results demonstrated that neither a tetramer nor a hexamer (all PO) oligodeoxynucleotide complementary to 514 RNA was able to induce RNase H cleavage in this nuclear extract system. When PO/PC-containing pentadecamers containing either two or four consecutive PO linkages were used, a low but readily detectable level of RNase H cleavage occurred. In contrast, a pentadecamer containing six consecutive PO linkages elicited complete RNase H cleavage of the substrate RNA. Note that the six

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PO nucleotides in this oligomer (oligomer M in Table 2) are identical in sequence to the RNase H-inactive hexamer (oligomer J), from which it is inferred that the potency of the pentadecamer reflects its increased hybrid
05 stability with 514 RNA owing to the additional nine complementary nucleotides.

Similar tests were performed with mixed PO/PC pentadecamers complementary to a different site in 514 RNA (i.e., nucleotides 463-477). These tests revealed an
10 effect of the number of PO linkages on RNase H cleavage similar to that described above.

Additional variants of mixed PO/PC oligomers were also tested. Pentadecamer PO/PC oligomers with five or six consecutive PO linkages at either the extreme 5' or
15 3' end were highly effective in eliciting RNase H cleavage. A PS/PC pentadecamer with six consecutive PS linkages at the extreme 5' end (oligomer P in Table 2) was only partially active. Comparison of these results with lanes 2 and 3 in Figure 4B confirms the above-
20 described results showing that all-PS oligomers are less effective than all-PO oligomers in eliciting RNase H cleavage. RNase H cleavage of 514 RNA was also observed with a 15-mer containing nine consecutive PM or PB linkages followed by six PO-linked nucleotides (oligomers
25 Q and R in Table 2).

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CLAIMS

1. A method of site-specific alteration of a target RNA segment of an RNA molecule, comprising the steps of:
 - a) combining the RNA molecule with a mixed phosphate backbone oligonucleotide which is complementary to all or a portion of the RNA molecule, in the presence of RNase H, the mixed phosphate backbone oligonucleotide including an internal segment which activates RNase H and two flanking nucleotide sequences which are unable to activate RNase H, one of the two flanking nucleotide sequences being present on either side of the internal segment, under conditions appropriate for hybridization of complementary nucleotide sequences and activation of RNase H, thereby excising the target RNA segment, creating a gap in the RNA molecule and producing an interrupted RNA molecule which has two segments; and
 - b) contacting the interrupted RNA molecule with an appropriate ligase, under conditions appropriate for joining the two segments of the interrupted RNA molecule by the ligase, thereby producing an altered RNA molecule.
2. The method of Claim 1 wherein the mixed phosphate backbone oligonucleotide is a mixed phosphate backbone oligodeoxynucleotide; the internucleoside bridging phosphate residues of the internal segment are unmodified phosphates and the internucleoside

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bridging phosphate residues of the flanking sequences are modified phosphate residues.

- 05 3. The method of Claim 2 wherein the bridging phosphate residues of the flanking sequences are modified of: methyl phosphonates, phosphoromorpholidates, phosphoropiperazidates, and phosphoramidates.

- 10 4. The method of Claim 1 wherein the mixed phosphate backbone oligonucleotide is a mixed phosphate backbone oligodeoxynucleotide; the internucleoside bridging phosphate residues of the internal segment are modified phosphates and the internal segment bridging phosphate residues of the flanking sequences are modified phosphates.

- 15 5. The method of Claim 4 wherein the internucleoside bridging phosphate residues of the internal segment are modified phosphates which are phosphorothioates and the internucleoside bridging phosphate residues of the flanking sequences are modified phosphates consisting of: methyl phosphonates, phosphoromorpholidates, phosphoropiperazidates, and phosphoramidates.

- 20 6. A method of producing a selected polypeptide comprising expressing an altered RNA molecule produced by the method of Claim 1.

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- 05 7. A method of producing a selected polypeptide encoded by an altered RNA molecule, comprising the steps of:
- a) providing an altered RNA molecule produced by a method of site-specific alteration of a target RNA segment of an RNA molecule; and
 - b) expressing the altered RNA molecule provided in step a).
- 10 8. A method of site-specific alteration of Claim 1, further comprising replacing the target RNA segment excised in step a) by introducing a segment of replacement nucleotides into the gap in the RNA molecule and b) linking the nucleotide present at each end of the segment of replacement nucleotides to the respective nucleotide present at the gap
- 15 created in the RNA molecule.
9. A mixed phosphate backbone oligonucleotide useful in the method of Claim 1.
- 20 10. A mixed phosphate backbone oligonucleotide consisting essentially of an internal segment of deoxynucleotides which activates RNase H and two modified nucleotide sequences which do not activate RNase H, in which the two modified nucleotide sequences flank the internal segment, one on each side of the internal segment.
- 25 11. The mixed phosphate backbone oligonucleotide of Claim 10 wherein the internucleoside bridging phosphate residues of the internal segment are

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- unmodified phosphates and the internucleoside bridging phosphate residues of the two modified nucleotide sequences are modified phosphates selected from the group consisting of: methyl phosphonates, phosphoromorpholidates, phosphoropiperazidates, and phosphoramidates.
- 05
12. The mixed phosphate backbone oligonucleotide of Claim 10 wherein the internucleoside bridging phosphate residues of the internal segment are modified phosphates which are phosphorothioates and the internucleoside bridging phosphate residues of the two modified nucleotide sequences are modified phosphates selected from the group consisting of: methyl phosphonates, phosphoromorpholidates, phosphoropiperazidates, and phosphoramidates.
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- 15

[illegible]

Fig. 1

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I G A A - A A U - A U C - A U C - G G U - G U U - U C C - U A U

II Am - TmAmGm - Ts AsGs - Cs CsAs - CmAmAm - Am

IIIa G A A - A A U - A U C - G U U - U C C - U A U

IIIb Am - TmAmGm - Ts AsGs - Cs CsAs - CmAmAm - Am

IVa p - A U C - U U U - G G U

IVb CmUmUm - TmTmAm - TmAmGm - AmAmAm - CmCmAm - CmAmAm - AmGmGm - AmUmAm

V G A A - A A U - A U C - U U U - G G U - U C C - U A U

CmTmTm - TmTmAm - TmAmGm - AmAmAm - CmCmAm - CmAmAm - AmGmGm - AmTmAm

Fig. 2

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 91/00968

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate 'all') *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ⁵ : C 12 N 15/10, C 12 N 15/11, C 07 H 21/04, C 12 P 21/02		
II. FIELDS SEARCHED		
Minimum Documentation Searched *		
Classification System	Classification Symbols	
IPC ⁵	C 12 N, C 07 H	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched *		
III. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. **
X	EP, A, 0260032 (AJINOMOTO) 16 March 1988 see abstract; page 2, lines 17-54; page 24, lines 45-51; claim 1	1,6-10
Y	--	2,3,11
Y	WO, A, 8905358 (UNIVERSITY OF IOWA RESEARCH FOUNDATION) 15 June 1989 see page 6, lines 25-29; example 2	2,3,11
P,X	WO, A, 9015065 (GILEAD SCIENCES) 13 December 1990 see page 5, line 12 - page 9, line 9; formula III; page 6, claims	9-12

<p>* Special categories of cited documents: 10</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
2nd May 1991	13. 06. 91	
International Searching Authority	Signature of Authorizing Officer	
EUROPEAN PATENT OFFICE	D. VORBILO NURIA TORIBIO	

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9100968
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0260032	16-03-88	AU-A- 1677688 JP-A- 63183599	01-12-88 28-07-88
WO-A- 8905358	15-06-89	AU-A- 2782989 EP-A- 0348458 JP-T- 2502516	05-07-89 03-01-90 16-08-90
WO-A- 9015065	13-12-90	None	

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